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Species identification of Chilean *Artemia* populations based on mitochondrial DNA RFLP analysis

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ABSTRACT

Aim Two species of the brine shrimp, namely *Artemia franciscana* Kellogg and *A. persimilis* Piccinelli and Prosdocimi, inhabit Chile. Most studies so far have shown that *A. franciscana* is the most widely distributed species in Chile, with *A. persimilis* present only in Chilean Patagonia. In general, there is good agreement between morphological and genetic comparisons of Chilean populations with respect to species discrimination. However, a number of results indicate an overlap with some populations tending to diverge from *A. franciscana* and/or resembling *A. persimilis*. Prior to the mid 90's the use of DNA markers in *Artemia* was rather limited, despite their successful application in numerous other species. In this study, we investigate whether the conclusions drawn from traditional comparative tools are congruent with the pattern of genetic divergence depicted by DNA analysis at the mitochondrial level.

Location Eight sites in Chile and two reference samples of *A. franciscana* and *A. persimilis* from San Francisco Bay (USA) and Buenos Aires (Argentina), respectively.

Methods Restriction fragment length polymorphism (RFLP) analysis of a 535 bp segment of the mitochondrial 16S rRNA gene with nine restriction enzymes in 240 individuals.

Results No haplotype was shared between the two species. Five restriction enzymes produced species-specific patterns, enabling the unambiguous assignment of populations to species. Very high (100%) bootstrap values supported the clustering of haplotypes in two groups corresponding to the two species. The two species were clearly differentiated with average sequence divergence of 12.3%. High genetic differentiation was also found among conspecific populations of *A. franciscana* with an F_{ST} estimate of 91%.

Main conclusions The mitochondrial DNA (mtDNA) results of this study show a broadly similar pattern to those of previous allozyme and nuclear DNA analyses, with the two New World species appearing as highly divergent. The presence of *A. persimilis* in southern Chile (Chilean Patagonia) was confirmed. Hence, a species previously regarded as geographically restricted mainly to Argentina, appears to have expanded its range. Populations of *A. franciscana* appear highly structured with a level of inter-population genetic differentiation much higher for mtDNA than previously reported with allozymes. Clustering of these populations does not follow a clear geographic pattern. The identification of population-specific genetic markers for *A. persimilis* and *A. franciscana* will help to tackle further aspects of the speciation patterns of these species.

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INTRODUCTION

The genus of *Artemia* is regarded as a complex of bisexual species and parthenogenetic forms inhabiting hypersaline waters throughout the world, with the exception of the Antarctic (Triantaphyllidis *et al.*, 1998; Van Stappen, 2002). Asexual species are likely to have diverged from an ancestral form living in the Mediterranean area some 5.5 Ma (Abreu-Grobois, 1987) and currently seven bisexual species and several parthenogenetic types (found only in the Old World) are recognized (Gajardo *et al.*, 2002). Two bisexual species, namely *A. franciscana* and *A. persimilis*, are found in the New World. The former is widely distributed over numerous localities in North, Central and South America, including Chile, whilst *A. persimilis* was until very recently believed to be confined to some localities in Argentina (Triantaphyllidis *et al.*, 1998). *Artemia* populations from most of the Chilean sites listed by Van Stappen (2002) have been characterized at a number of levels, such as morphology (Gajardo *et al.*, 1998), karyology (Colihueque & Gajardo, 1996; Gajardo *et al.*, 2001a), allozymes (Gajardo & Beardmore, 1993; Gajardo *et al.*, 1995, 1999) and cross-fertility tests (Gajardo *et al.*, 2001b). These studies have demonstrated that *A. franciscana* is the most widely distributed species in Chile, either in coastal or inland lagoons and salt lakes between regions I and VI (20–33°S). These habitats are scattered throughout the country and some of them are characterized by extreme environmental conditions like those found in the Atacama Desert, reputedly one of the driest areas in the world (Gajardo *et al.*, 1992; Gajardo & Beardmore, 1993). Likewise, the presence of *A. persimilis* in the Chilean Patagonia (region XII, below 50°S), which is also a unique environment (Gajardo *et al.*, 1998, 1999), redraws its distribution map and challenges its ability to disperse or colonize habitats further north, occupied by *A. franciscana*.

Compared with *A. franciscana*, *A. persimilis* seems to be closer to the ancestral group of species that evolved in the Mediterranean basin and, consequently, it may be regarded as more primitive (Abreu-Grobois, 1987; Badaracco *et al.*, 1987; Browne & Bowen, 1991). Both species evolved in geographical isolation, therefore the expansion of *A. persimilis* into Chile represents a unique opportunity to test some predictions relating to allopatric speciation (see Gajardo *et al.*, 2001b), in particular the type of reinforcement of reproductive isolation. *Artemia persimilis* differs from *A. franciscana* by the presence of one extra pair of chromosomes ($2n = 44$) and a lower number of chromocentres (*Alu*I-type of repetitive DNA) (Barigozzi, 1974; Badaracco *et al.*, 1987). The large amount of repetitive DNA in *A. franciscana* is probably because of amplification of *Alu*I sequences by unequal crossing over (Badaracco *et al.*, 1987). Molecular DNA tools have been widely applied in different organisms in the past decades (see Avise, 1994, 1999 and references therein). All are based ultimately on the use of genetic similarities and differences to measure relatedness. Although they have greater power than 'traditional' techniques such as allozymes (Estoup *et al.*, 1998), inferences from such assays cannot be readily accepted before more data are gathered regarding how DNA sequences evolve and how observed patterns are related to fitness traits (Amos, 1999). Over the last decade, modern techniques and new molecular markers have been increasingly used in *Artemia* research (Abatzopoulos *et al.*, 1997; Triantaphyllidis *et al.*, 1997; Sun *et al.*, 1999; Abatzopoulos *et al.*, 2002a,b). Their application, in combination with complementary information from other techniques, makes them particularly useful in providing better descriptions and interpretations of biological diversity (e.g. Triantaphyllidis *et al.*, 1997; Abatzopoulos *et al.*, 2002b). The mitochondrial DNA (mtDNA) offers multiple advantages for evolutionary studies in closely related species because of its high rate of evolution, maternal mode of inheritance and lack of recombination among others (Moore *et al.*, 1994). Its mutation rate is about five to 10 times higher compared with single-copy nuclear DNA. Very preliminary attempts based on mtDNA restriction fragment length polymorphism (RFLP) analysis, were ventilated in the early 1990s for determining evolutionary divergence in *Artemia* (Browne & Hoopes, 1990; Browne & Bowen, 1991). Additionally, the complete sequence of *A. franciscana* mtDNA, published almost a decade ago (Valverde *et al.*, 1994), served as a valuable tool for initiating research on various fields such as population structure and molecular ecology.

Studies on Chilean *Artemia* populations show a broadly good correspondence between morphological and genetic (mostly allozymic) traits (see Gajardo & Beardmore, 2001) with regard to species demarcation. However, there are some populations that tend to diverge from *A. franciscana* and/or resemble *A. persimilis*. In this research effort, we investigate whether the conclusions drawn from traditional comparative tools (Gajardo & Beardmore, 2001) are congruent with the pattern of genetic divergence depicted by RFLP analysis of mtDNA. In particular, we have tried to identify population-specific DNA markers in order to evaluate (i) the species status of Chilean *Artemia* populations (when compared with reference samples of *A. franciscana* and *A. persimilis*), (ii) their genetic structure and (iii) their phylogeographic pattern. Last but not least, comparing the mtDNA results with those previously obtained by characterizations based on morphology, allozymes and chromocentres is important in

formulating more robust hypotheses regarding biodiversity, genetic/phenotypic divergence and speciation processes in *Artemia*.

MATERIALS AND METHODS

Adult *Artemia* individuals were collected from eight different populations in Chile (Table 1, Fig. 1). Four of these populations are known to belong to *A. franciscana* (Iquique, Llamara, Cejas and Los Vilos), one of them to *A. persimilis* (Torres del Paine), whereas for Chaxas and El Convento the species status has not been identified yet. Pichilemu population has been a controversial case (see Table 1 and Discussion section). Samples from San Francisco Bay, USA and Buenos Aires, Argentina strains were used as references for *A. franciscana* and *A. persimilis* species, respectively.

MtDNA RFLP analysis

DNA was extracted from 240 individuals using the CTAB method described in Hillis *et al.* (1996). Part of the 16S rRNA gene was amplified using the universal primers L_{2510} and H_{3080}, (Palumbi *et al.*, 1996). PCR reactions were set up in a volume of 75 μL and were composed of 45 pmoles of each primer, 0.15 mM of each dNTP, 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 4 mM $MgCl_2$ and 1.5 U of *Taq*-DNA polymerase (Invitrogen, Cazlsband, California, USA). Approximately 100 ng total genomic DNA in Tris-EDTA was added to this. The following amplification cycles programme was used: initial denaturation at 95 °C for 4 min, 33 cycles consisting of 94 °C for 1 min, 50 °C for 50 s, 72 °C for 1 min. PCR products (4–7 μL) were digested with nine restriction endonucleases, electrophoretically separated in 1.5% agarose gel, stained with ethidium bromide, visualized and photographed under UV light. The following restriction enzymes were used: *Alu*I, *Bfa*I, *Dde*I, *Dpn*II, *Hae*III, *Msp*I, *Not*I, *Rsa*I and *Taq*^{a}I. For molecular weight size standard a 100 bp ladder (New England BioLabs, Beverly, Massachusetts, USA) was used.

Data analysis

A specific letter was used to identify distinct, single endonuclease restriction patterns. Each individual was assigned a multi-letter code that described its composite mtDNA genotype. The raw data were fragment profiles, but we inferred site differences among haplotypes from changes in fragment profiles as these could be accounted for by the gain or loss of particular restriction sites. Site inference was aided by the published sequence of the whole mtDNA of *A. franciscana* (GI: 5835051, Valverde *et al.*, 1994). Haplotype and nucleotide diversity values within a population, and nucleotide divergence between haplotypes and populations were computed using the statistical package REAP 4.0 (McElroy *et al.*, 1991). The genetic software Arlequin 2.0 (Schneider *et al.*, 2000) was used to test for population differentiation based on haplotype frequencies (using 10,000 randomizations) and to evaluate the degree of population genetic structure (F_{ST}) through analyses of molecular variance (Excoffier *et al.*, 1992). F_{ST} values were computed including the distance matrix of the haplotypes. Throughout the analysis corrections for multiple comparisons were applied using sequential Bonferroni correction (Rice, 1989). Trees based on distance or parsimony methods were drawn and bootstrap values were estimated with the PHYLIP 3.57c (Felsenstein, 1995) computer package. Correlation of pairwise nucleotide divergence over geographical distances for all pairs (i) of examined *Artemia* populations and (ii) of examined *A. franciscana* populations was tested with the Mantel permutation procedure (Mantel, 1967) available in GENETIX 4.0 (Belkhir, 1999).

Table 1 List of *Artemia* populations used in this study

Locality	Coordinates	Abbreviation	Species status	References
San Francisco Bay (USA)	37°28′N 122°30′W	SFB	*A. franciscana*	ARC code No. 1258
Iquique (Chile)	20°40′S 70°15′W	IQU	*A. franciscana*	Gajardo *et al.* (1998)
Llamara (Chile)	21°18′S 69°37′W	LLA	*A. franciscana*	Gajardo *et al.* (1998)
Chaxas (Chile)	22°47′S 67°58′W	CHA	*	
Cejas (Chile)	23°02′S 68°13′W	CEJ	*A. franciscana*	Gajardo *et al.* (1998)
Los Vilos (Chile)	31°58′S 71°25′W	LVI	*A. franciscana*	Gajardo *et al.* (1998)
El Convento (Chile)	33°52′S 71°44′W	CON	*A. franciscana**	De los Ríos (2000)
Pichilemu (Chile)	34°25′S 72°10′W	PIC	*A. franciscana*	†
Torres del Paine (Chile)	50°29′S 73°45′W	TPA	*A. persimilis*	Gajardo *et al.* (1999)
Buenos Aires (Argentina)	34°30′S 58°20′W	BAI	*A. persimilis*	ARC code No. 1321

ARC, *Artemia* Reference Center, Ghent, Belgium.

*Unknown or non-verified.

†The species status of this population is controversial (see Gajardo *et al.*, 1995; Colihueque & Gajardo, 1996; Gajardo *et al.*, 1998, 2001a,b).

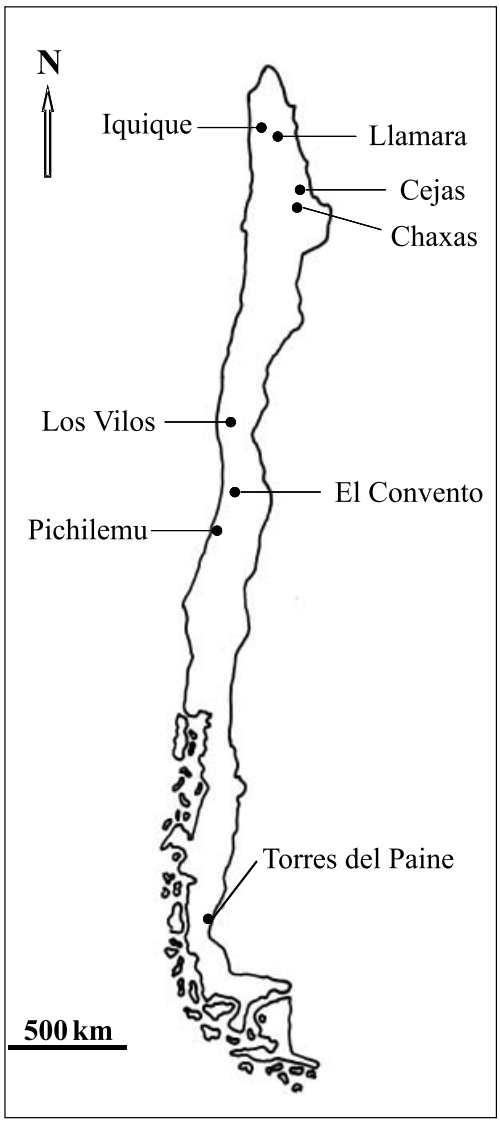

Figure 1 Sampling sites of Chilean *Artemia* populations.

Table 2 Fragment size estimates (base pairs) of restriction patterns observed in the 16S rRNA region among the *Artemia* populations analysed

*Alu*I	A^F	B^P	*Dpn*II	$A^{F,P}$	$B^{F,P}$	*Not*I	A^F	B^P
340	–		230	–	–	535	–	
260		–	180	–		300		–
195	–		125	–	–	235		–
155		–	110		–			
120		–	70		–			
*Bfa*I	A^F	$B^{F,P}$	*Hae*III	A^F	B^P	*Rsa*I	A^F	$B^{F,P}$
535		–	470	–		535		–
480	–		235		–	330	–	
55	–		235		–	205	–	
			65	–	–			
*Dde*I	A^F	B^P	*Msp*I	A^F	$B^{F,P}$	*Taq*^{a}I	A^F	B^P
385		–	535	–		260	–	
360	–		295		–	250		–
150	–	–	240		–	170		–
25	–					120	–	
						55	–	–
						40	–	–
						40	–	
						20	–	–

F indicates pattern present in *A. fransciscana*.
P indicates pattern present in *A. persimilis*.

RESULTS

The size of the PCR-amplified mtDNA fragment was 535 bp. Routine screening of all populations revealed a total of 20 sites corresponding to 82 nucleotides. The fragment patterns generated by each enzyme are given in Table 2. In total, seven composite mtDNA genotypes were found (Table 3). The maximum number of observed haplotypes within population samples was two. Two of seven haplotypes (FRANC3, PERS3) were private, i.e. population-specific.

The UPGMA dendrogram resulting from the distance matrix between haplotypes revealed two clusters (Fig. 2). Cluster A is comprised of three haplotypes (including those of the BAI *A. persimilis* reference sample) and cluster B of four haplotypes (including those of the SFB *A. franciscana* reference sample). The clustering of the haplotypes in two different groups was also supported by a bootstrap value of 100%. No other grouping was highly supported. Pairwise sequence divergence estimates between these haplotypes were *c.* 1.1% and 1.5% within clade A (*A. persimilis*) and clade B (*A. franciscana*) respectively, while 12.3% between the two species.

The asymmetrical distribution of population nucleotide divergence values (Table 4) is clear evidence of the level of genetic differentiation between the two species. This is also confirmed by the UPGMA tree constructed based on pairwise divergence values, with the population samples separating in the two species (Fig. 2). Accordingly, the species status of the Chilean populations can be deduced: populations PIC and TPA are assigned to *A. persimilis* whereas populations IQU, LLA, CHA, CEJ, LVI and CON are assigned to *A. franciscana*. Within the *A. franciscana* cluster, the northernmost population IQU seems to be the most divergent from the rest and it groups with the reference sample (SFB).

Haplotype diversity values varied from 0.00 in most population samples, to 0.51 (in CHA) (Table 3). Haplotype and nucleotide diversity values were higher for *A. persimilis* population samples (Table 3). Five restriction enzymes (*Alu*I, *Dde*I, *Hae*III, *Not*I and *Taq*^{a}I) produced species-specific patterns. Three restriction enzymes (*Bfa*I, *Dpn*II and *Rsa*I) were polymorphic for *A. franciscana* and two (*Dpn*II and *Msp*I) for *A. persimilis*. No composite haplotype was shared between individuals from the two species.

Tests of population differentiation based on haplotype frequencies showed that, globally, results were statistically significant ($P < 0.001$). In addition, only three of 45 pairwise

Table 3 Composite genotypes (haplotypes), haplotype frequencies, sample sizes (n), haplotype diversity (h), with SE and nucleotide diversity percentage (π) of the studied *Artemia* sp. populations, based on restriction sites data. The enzymes used were in the following order: *Alu*I, *Bfa*I, *Dde*I, *Dpn*II, *Hae*III, *Msp*I, *Not*I, *Rsa*I and *Taq*^{a}I. For abbreviations of population names see Table 1

		A. franciscana							*A. persimilis*		
		SFB	IQU	LLA	CHA	CEJ	LVI	CON	PIC	TPA	BAI
Franc1	AAABAAAAA				0.48	1.00	1.00				
Franc2	AAAAAAAAA	0.95	1.00								
Franc3	ABAAAAAAA	0.05									
Franc4	AAABAAABA			1.00	0.52			1.00			
Pers1	BBBABABBB								1.00		0.26
Pers2	BBBBBABBB									0.32	0.74
Pers3	BBBBBBBBB									0.68	
n		23	24	25	25	27	26	20	25	22	23
h		0.085	0.0	0.0	0.510	0.0	0.0	0.0	0.0	0.444	0.394
SE		0.055	0.0	0.0	0.015	0.0	0.0	0.0	0.0	0.053	0.063
π (%)		0.079	0.0	0.0	0.442	0.0	0.0	0.0	0.0	0.374	0.355
Mean h					0.085					0.279	
SE					0.005					0.019	
Mean π (%)					0.074					0.252	

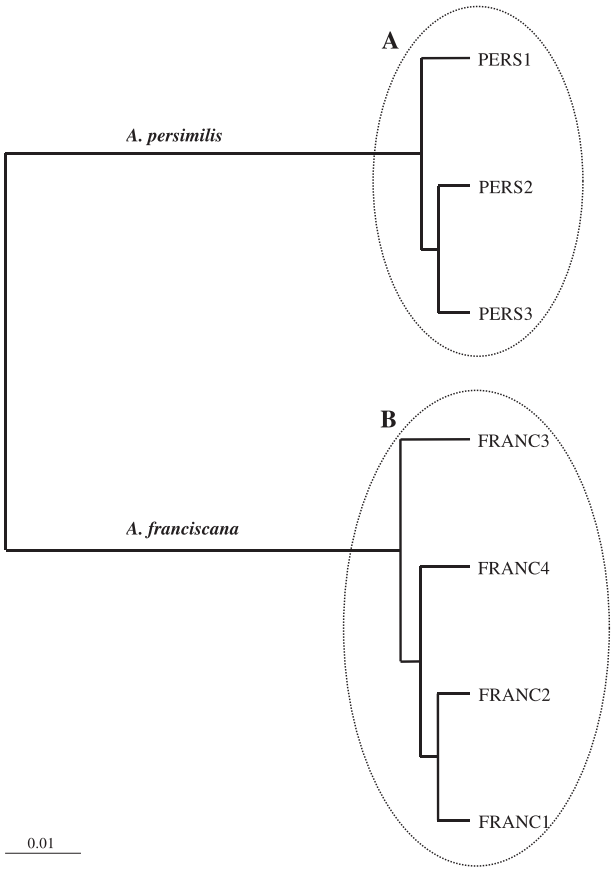

Figure 2 UPGMA phylogenetic tree clustering the seven *Artemia* mtDNA composite haplotypes described in Table 2.

comparisons (with or without Bonferroni correction) between populations were not significant ($P > 0.05$), i.e. SFB vs. IQU, LLA vs. CON and CEJ vs. LVI. Mantel tests on correlation of pairwise nucleotide divergence to geographical distances between pairs of all examined *Artemia* populations and all *A. franciscana* populations were not significant ($P = 0.30$ and $P = 0.29$, respectively).

Table 4 Mean nucleotide divergence values between *Artemia* populations studied. For abbreviations of population names see Table 1

Population	1	2	3	4	5	6	7	8	9	10
SFB	–									
IQU	0.000	–								
LLA	0.018	0.018	–							
CHA	0.011	0.011	0.002	–						
CEJ	0.009	0.008	0.008	0.002	–					
LVI	0.009	0.008	0.008	0.002	0.000	–				
CON	0.018	0.018	0.000	0.002	0.008	0.008	–			
PIC	0.123	0.124	0.124	0.126	0.133	0.133	0.124	–		
TPA	0.136	0.137	0.109	0.111	0.117	0.117	0.109	0.013	–	
BAI	0.128	0.129	0.108	0.110	0.117	0.117	0.108	0.005	0.004	–

Analysis of the partitioning of the total amount of polymorphism with AMOVA gave a value of $F_{ST} = 98.7\%$. However, 91% of the genetic polymorphism was due to differences between the two identified species, whereas only 7.7% was due to differences among populations within the species. The remaining 1.3% accounted for polymorphism within populations within species. The F_{ST} value for *A. franciscana* population samples was also very high (91%).

DISCUSSION

The brine shrimp *Artemia* is a highly favourable organism for evolutionary studies, as it has a range of key factors driving speciation not very often seen in other organisms (Gajardo *et al.*, 2002). The presence in Chile of the two New World species, *A. franciscana* and *A. persimilis*, offers an interesting case of evolution in progress as these two species, which evolved in isolation, could eventually come into contact.

According to the allopatric speciation paradigm, this corresponds to the second phase of the speciation process, when reproductive isolation mechanisms are reinforced. As a first step towards getting a clearer picture of the level of divergence between the two species, this study aimed at confronting previous data on the population structure of Chilean *Artemia* to that revealed by the analysis of RFLPs of mtDNA. In particular, focus was placed on mtDNA variability within populations from different geographic locations in Chile as a way to establish their levels of genetic divergence, and to verify if both species are present in Chile as previously suggested by morphological (Gajardo *et al.*, 1998), protein electrophoresis (Gajardo & Beardmore, 1993; Gajardo *et al.*, 1995, 1999) and karyological data (Colihueque & Gajardo, 1996; Gajardo *et al.*, 2001a; Gajardo & Beardmore, 2001). Five restriction enzymes were found to give species-specific patterns, which make them reliable markers for this purpose. Two reference samples of *A. franciscana* and *A. persimilis* were used for comparison purposes. Among the seven haplotypes produced, four matched with the former species whilst three with the latter with 100% bootstrap support (Fig. 2).

The analysis concerning the structure of *Artemia* populations in Chile suggests that six of eight Chilean populations studied, are grouped with the *A. franciscana* reference sample (Fig. 3). The species status was already known for the IQU, LLA, CEJ and LVI populations. No genetic information was available so far for the CHA and CON populations. Therefore, this demonstrates the power of molecular markers for a simple and fast (initial) genetic identification of samples. The remaining two populations (PIC and TPA) show haplotypes, which are closely related to those of the *A. persimilis* reference sample. This is not unexpected, in the light of previous electrophoretic and karyological results. TPA generally shares genomic characteristics with *A. persimilis* (diploid number and con-specific Nei's genetic distances, Colihueque & Gajardo, 1996; Gajardo *et al.*, 1999) with the only exception, that of its chromocentre frequencies, which are typical of *A. franciscana* (Gajardo *et al.*, 2001a). However, the species status of the PIC population has been controversial. It has been cytogenetically (Colihueque & Gajardo, 1996; Gajardo *et al.*, 2001a) and morphometrically (Gajardo *et al.*, 1998) characterized as *A. persimilis*, whereas it is electrophoretically closer to *A. franciscana* (Gajardo *et al.*, 1995). This mixture of genomic characteristics from two different species in one or several individuals is often an indication of probable hybridization (Gajardo *et al.*, 2002) and of presence of a hybrid zone. The geographical proximity between CON and PIC populations, which belong to the two different species, and the fact that the PIC population breeds successfully with both *A. franciscana* and *A. perisimilis* individuals (Gajardo *et al.*, 1998; Gajardo *et al.*, 2001b) support this view.

Hence, this work confirms the presence of both species in Chile, with *A. franciscana* ranging between 20 and 34°S and *A. persimilis* confined to southern Chile. According to Gajardo *et al.* (1999), the presence of *A. persimilis* in southern Chile would be explained by the fact that the Andes, the natural barrier between Chile and Argentina, show lower altitudes as latitude increases. Therefore, chances for dispersion by flamingos are greater than in any other place along the southern limits of the mountain range. However, this hypothesis cannot explain the PIC population case. Further studies are needed to clarify the dispersal pattern of *A. persimilis* in Chile.

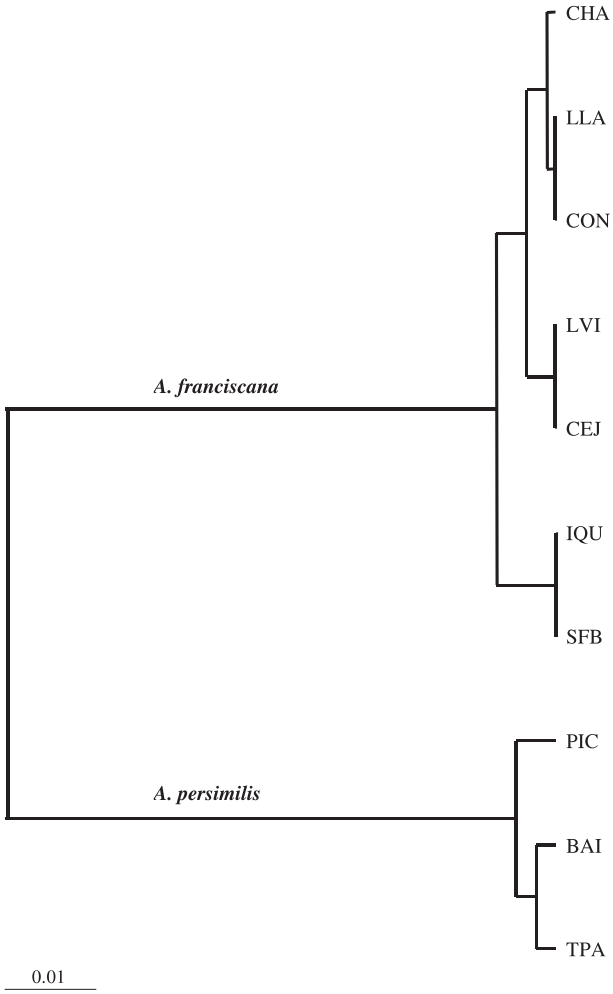

Figure 3 UPGMA phylogenetic tree clustering all *Artemia* populations according to the distance matrix of sequence divergence, based on restriction sites data. For abbreviations of population names see Table 1.

The high sequence divergence (12.3%) observed at the mtDNA level between the two species, agrees with previously reported nuclear DNA data such as those provided by allozymes and RAPD's (Nei's D genetic distance higher than 1, Badaracco *et al.*, 1995), diploid number ($2n = 44$ in *A. persimilis* vs. $2n = 42$ in *A. franciscana*) and mean chromocentre numbers (Badaracco *et al.*, 1987; Gajardo *et al.*, 1995, 2001a; Gajardo & Beardmore, 2001). This level of interspecific differentiation is also in agreement with values of divergence between bisexual and parthenogenetic *Artemia* calculated by Perez *et al.* (1994), and with the results of

Remigio *et al.* (2001) who sequenced the same mtDNA region in species of the phylogenetically close *Parartemia* genus. These researchers found estimates of inter-specific divergence ranging from 11.3 to 20.7%. Estimating the time of divergence of two species based on sequence data, using the notion of molecular clock, requires the acceptance of certain assumptions, on which there is not always agreement (Nei, 1987). Based on mitochondrial rDNA standard calibrations (Lynch & Jarrell, 1993), the two species must have diverged more than 50 Ma. However, on the basis of elevated rates of evolution discovered in halophilic species (Remigio *et al.*, 2001), this divergence estimate should only be regarded as an upper limit.

It is also worth noting that mean haplotype and per cent nucleotide diversity values in the mtDNA fragment studied were higher for *A. persimilis* (0.279 ± 0.019; 0.252) than for *A. franciscana* (0.085 ± 0.005; 0.074). The latter is considered as a species in evolutionary expansion, a fact also supported by the presence of high levels of variability in coding and non-coding genomic regions (Gajardo *et al.*, 2002). However, the higher haplotype and nucleotide diversity observed in *A. persimilis* could be explained by its primitive status, i.e. it originated at an earlier stage than *A. franciscana* (Gajardo *et al.*, 2001a, 2002). Hence, one would expect a greater number of mutations to have accumulated in *A. persimilis* mtDNA than in *A. franciscana*. However (and not exclusive to the previous statement) one should not forget the generally low rates of mutations in the 16S rRNA gene, which might account for the absence of variability in six of 10 studied populations and the inherent limitations of the RFLP technique to detect the total variability present in the samples.

Results of this study also confirm the general trend of *A. franciscana* to build high inter-population differences, which has been also reported for the Chilean populations by protein electrophoresis (Gajardo *et al.*, 1995). The restriction enzyme analysis produced higher F_{ST} values (91%) than those reported by electrophoresis (23%; G. Gajardo, unpublished results), indicating that the observed variation is by far because of differences among populations, a feature not fully detected by allozymes. However, this differentiation does not follow an 'isolation by distance' pattern (as evident by the absence of significance in Mantel tests on the correlation of geographic to genetic distances of populations) or any other clear geographic pattern. For example, in the UPGMA dendrogram (Fig. 3), populations CON and LLA cluster together, although there are other populations geographically closer. Most populations are fixed for different, albeit phylogenetically close, haplotypes (only one to three sites different). Therefore, the resulting grouping of populations is probably because of the fast lineage sorting of mtDNA haplotypes, caused by the reduced effective population size of the mtDNA molecule (Birky *et al.*, 1983). However, the importance of founding events must also be stressed (Gomez *et al.*, 2002). After the initial founding event, a rapid increase in population size occurs following colonization. With the local adaptation ensuing, migrants would have very little effect on the level of genetic differentiation between populations. This is probably the reason neighbouring populations are fixed for different haplotypes and share none. Indeed, only one Chilean *A. franciscana* population (CHA) of six shows haplotypic polymorphism.

The population-specific markers and the corresponding highly structured *A. franciscana* population organization are some of the most relevant aspects of the mtDNA analysis performed in this study. This shows that mtDNA markers are more informative than allozymes in the case of *Artemia*. As already mentioned, the power of molecular markers needs to be evaluated, and a way to do that is to compare their level of resolution with other less direct DNA analyses. Reliability and resolution power of mtDNA markers obtained in this work have been proved in such a way.

The population-specific haplotypes identified in this work will help explore and monitor the geographic expansion of *A. persimilis* in Chile and its outcome. At the same time, they will facilitate the analysis of laboratory cross-breeding experiments, aimed at evaluating the possibility of hybridization and the levels of reproductive isolation between both species. Gajardo *et al.* (2001b) have suggested that the apparent lack of efficient mechanisms of reproductive isolation between both species, despite significant genetic differences between them, would follow a trend often seen in other anostracans. This is likely to occur when formerly allopatric populations come into contact, a requirement according to the allopatric speciation paradigm, to reinforce isolation mechanisms.

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BIOSKETCHES

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